Matrix Metalloproteinase–9 in Pneumococcal Meningitis: Activation via an Oxidative Pathway

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In experimental bacterial meningitis, matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) contribute to brain damage. MMP-9 increases in cerebrospinal fluid (CSF) during bacterial meningitis and is associated with the brain damage that is a consequence of the disease. This study assesses the origin of MMP-9 in bacterial meningitis and how ROS modulate its activity. Rat brain-slice cultures and rat polymorphonuclear cells (PMNs) that had been challenged with capsule-deficient heat-inactivated *Streptococcus pneumoniae* R6 (hiR6) released MMP-9. Coincubation with either catalase, with the myeloperoxidase inhibitor azide, or with the hypochlorous acid scavenger methionine almost completely prevented activation, but not the release, of MMP-9, in supernatants of human PMNs stimulated with hiR6. Thus, in bacterial meningitis, both brain-resident cells and invading PMNs may act as sources of MMP-9, and stimulated PMNs may activate MMP-9 via an ROS-dependent pathway. MMP-9 activation by ROS may represent a target for therapeutic intervention in bacterial meningitis.

In bacterial meningitis, matrix metalloproteinases (MMPs) and reactive oxygen species (ROS), which are both produced as part of the host's immune response to bacteria, contribute to the pathogenesis of brain damage [1, 2]. MMPs are a family of zinc-dependent matrix-degrading enzymes that can disrupt the blood-brain barrier (BBB), a disruption that leads to extravasation of blood proteins, to brain edema, to cerebral hypoperfusion, and, ultimately, to neuronal damage [1, 3, 4]. ROS formation colocalizes with invading polymorphonuclear cells (PMNs) in the subarachnoid space and along penetrating cortical blood vessels and leads to oxidative alterations of the cerebral vasculature [2, 5]. Blockage of MMPs by specific MMP-inhibitors and neutralization of ROS by different antioxidants (such as *N*-acetylcysteine, *alpha* phenyl-*tert*-butyl nitrone, and desferoxamine) protect against brain damage in experimental meningitis [1, 6].

Regulation of the biological effects of MMPs occurs at the level of gene transcription, release, and enzyme activity [7]. Studies performed in a rat model of pneumococcal meningitis documented a 100–1000-fold transcriptional induction of MMP-3, -8, -9, -12, -13, and -14, in brain parenchymal tissue, but no change of MMP-2 or MMP-7 was observed. In cerebrospinal fluid (CSF) cells, mRNA of MMP-8 and MMP-9 was increased 10–100-fold, whereas MMP-2 and MMP-7 again remained unchanged [1]. On the protein level, MMP-9 appeared in CSF as early as 15 min after infection, which suggests release by brain-resident cells at this early stage of disease. Concentrations of MMP-9 peaked ~18 h after infection, when neutrophilic meningeal inflammation is prominent.

Furthermore, CSF concentrations of MMP-8 and MMP-9 are elevated in children with bacterial men-

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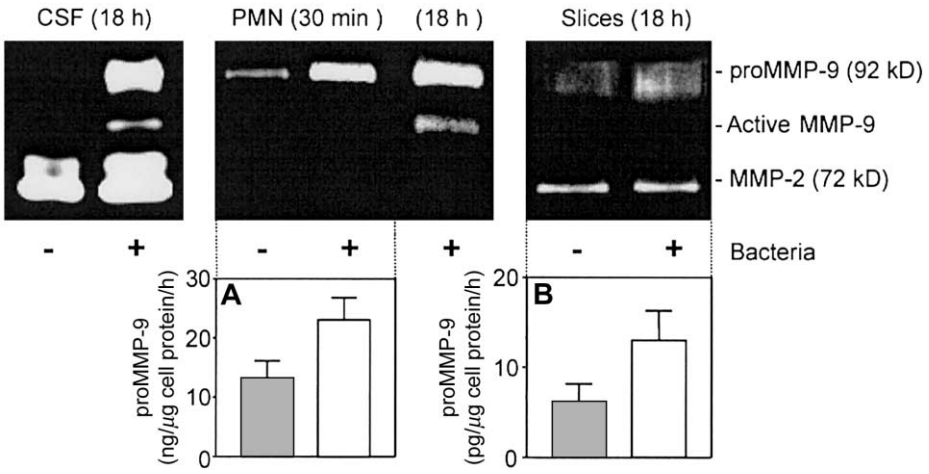

Figure 1. Zymography of cerebrospinal fluid (CSF), polymorphonuclear cell (PMN) supernatants, and organotypic brain-slice–culture supernatants. In CSF, matrix metalloproteinase (MMP)–2 was constitutively present, whereas proform MMP–9 (proMMP-9) and active MMP-9 were exclusively present in meningitis, at 18 h after infection. *A,* Rat PMN supernatants. After 30 min, supernatants stimulated with heat-inactivated *Streptococcus pneumoniae* R6 (hiR6; *white*) showed a 2-fold increase in release of proMMP-9, compared with unchallenged control supernatants *(gray)* (17.3 ± 2.9 vs. 9.6 ± 2.3 ng/mL; $n = 6$; $P < .001$). Whereas MMP-2 was absent at any time, active MMP-9 was found 18 h after infection. *B,* Brain-slice–culture supernatants. After 18 h, supernatants stimulated with hiR6 showed a 2-fold increase in release of proMMP-9 (5.1 ± 1.4 vs. 2.5 ± 0.7 ng/mL; $P < .01$), whereas MMP-2 remained unchanged. Per microgram of cell protein and per hour, PMNs released substantially more MMP-9 than did brain slices and therefore may act as a major source of MMP-9 in the CSF during bacterial meningitis.

ingitis and are significantly higher in those who develop neurological sequealae, compared with children who fully recover from the disease [3]. However, the exact origin of MMP-9 and the mechanism of MMP-9 activation in bacterial meningitis remain unclear.

MMP-9 is released in an inactive proform (proMMP-9) and must be processed to become biologically active [7]. The catalytic zinc molecule in proMMP-9 is sterically blocked in the prodomain by a cysteine residue (i.e., cysteine switch), a process that renders the enzyme inactive [8]. Activation of proMMP-9 occurs when the prodomain is cleaved by other proteases or when the cysteine switch is disrupted. Disruption can occur, at physiological concentrations of ROS, as a result of oxidation of the cysteine thiol group [8–10]. The results of the present study indicate that both invading PMNs and brain-resident cells are likely sources of MMP-9 in bacterial meningitis and that PMNs can activate proMMP-9 by an ROS-dependent mechanism.

MATERIALS AND METHODS

Materials. Catalase and L-methionine were obtained from Sigma; sodium azide was obtained from Merck; Percoll and Dextran T-500 were obtained from Amersham Pharmacia Biotech; and Neurobasal medium and B-27 supplement were obtained from Life technologies.

Heat-inactivated* Streptococcus pneumoniae *R6 (hiR6). The capsule-deficient *S. pneumoniae* strain R6 was grown overnight at 37°C in brain-heart infusion. The bacteria were centrifuged, were washed with sterile saline (0.9% NaCl), were resuspended in 5 mL Hanks' balanced salt solution (HBSS) (136.8 m*M* NaCl, 5.4 m*M* KCl, 0.3 m*M* Na_2HPO_4, 0.4 m*M* KH_2PO_4, 5 m*M* glucose [pH 7.4]), and were heat inactivated for 20 min at 80°C.

PMNs. Human PMNs and rat PMNs were isolated from peripheral blood by a combination of Dextran sedimentation and Percoll centrifugation. PMNs suspended in HBSS were stimulated with different concentrations of hiR6 at 37°C. After 30 min, culture supernatant was obtained by centrifugation and was analyzed immediately.

Organotypic tissue cultures (OTCs). OTCs, which retain the 3-dimensional architecture and local environment of brain cells (including neurons, glial cells, and other cells) to a greater extent than do dissociated cell cultures, are characterized by preserved tissue morphology and by cell-type–specific distribution. Brain-slice explants of the rat-brain cortex were prepared as described elsewhere [11]. Brain slices were cultured in neurobasal medium supplemented with B27, each slice in 1 insert, and were allowed to recover from explantation trauma for 11 days before bacterial stimulation was initiated. Slices were then overlaid with 10^7 cfu hiR6 in 100 μL Neurobasal medium. The stimulated slices were further maintained for 18 h at 37°C before analysis. Control cultures (without bacteria) were treated identically.

Animal model of pneumococcal meningitis. An infant-rat model of pneumococcal meningitis was used, as described elsewhere [1, 6]. In brief, 11-day-old Sprague-Dawley rats were

infected intracisternally with a defined inoculum of *S. pneumoniae* (serogroup 3). Eighteen hours later, 10–20 μL CSF was obtained by a puncture of the cisterna magna. CSF was centrifuged, and supernatant was used for further analysis.

Gelatin zymography. MMP-9 was quantified by gelatin zymography [1]. In this assay system, active and latent forms of MMP-9 show the same degree of gelatin digestion [12]. Ten microliters rat PMN supernatant, or 3 μL CSF, or 10 μL OTC supernatant was diluted with H_2O and 4× sample buffer (0.25 *M* Tris [pH 6.8], 10% SDS, 40% glycerol, 0.05% bromophenol blue), to a loading volume of 16 μL, and was electrophoresed in a 10% SDS polyacrylamide gel containing 0.1% gelatin (Novex). After electrophoresis for 2 h at 100 V, gels were incubated for 1 h in SDS-removing buffer (1% Triton X-100; 3 changes) and then were incubated for 12 h at 37°C with incubation buffer (10 m*M* $CaCl_2$, 50 m*M* Tris, 50 m*M* NaCl [pH 7.6]). The gels were stained with Coomassie brilliant blue (0.5% Coomassie, 30% methanol, 10% acetic acid). The gelatinolytic activity of MMP-9 was quantified by densitometric analysis (public domain, National Institutes of Health Image program) of the substrate lysis zone around 92 kDa. Purified human neutrophil MMP-9 (Calbiochem) was used as a standard. Under these conditions, samples were within the linear range of the standard curve.

MMP-9 activity. MMP-9 activity and total MMP-9 of human PMN supernatants were assessed by an MMP-9 assay system (Biotrak; Amersham Pharmacia Biotech). Samples were incubated in microtiter wells that had been precoated with anti–MMP-9 antibody, which binds both active and proMMP-9, and activity was measured by substrate cleavage. To measure total MMP-9 content, bound proMMP-9 was activated by aminophenylmercuric acetate before addition of the detection enzyme.

Statistical analysis. An analysis-of-variance test with Bonferroni post hoc correction for multiple comparisons was performed (Prism Software; GraphPad). $P < .05$ was considered significant. The results were expressed as mean ± SD.

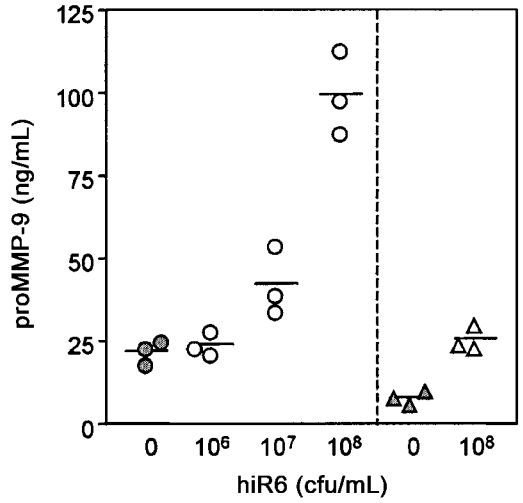

Figure 2. Release of proform matrix metalloproteinase-9 (proMMP-9) by human polymorphonuclear cells (PMNs). Exposure of 2×10^6 human PMNs/mL *(circles)* to increasing concentrations (10^6–10^8 cfu/mL) of heat-inactivated *Streptococcus pneumoniae* R6 (hiR6; *white*) resulted in dose-dependent release ($R^2 = 0.93$; $P < .0001$) of proMMP-9 into the supernatant, as quantified by gelatin zymography, compared with unstimulated control cultures *(gray).* Decreasing the number of PMNs (4×10^5/mL; *triangles*) resulted in a correspondingly smaller release of proMMP-9.

RESULTS

Origin of MMP-9 in bacterial meningitis. Zymography revealed an increase of proMMP-9 (92 kDa) and of active MMP-9 (83 kDa), in the CSF of rats that were suffering from pneumococcal meningitis, 18 h after infection (figure 1, *upper-left panel*). In contrast, levels of MMP-2 remained unchanged. In vitro, peripheral rat PMNs released proMMP-9 ⩽30 min after challenge with hiR6 (figure 1, *upper-middle panel,* and 1*A*). Similarly to the in vivo findings, active MMP-9 (83 kDa) was found 18 h after challenge with hiR6. Since proMMP-9 is not activated in the absence of cells, we conclude that proMMP-9 was released and subsequently was processed to its active form by the PMNs. In brain-slice cultures, which lack invading PMNs, proMMP-9 increased after hiR6 stimulation, a finding that indicates that brain-resident cells may also act as sources of MMP-9 in bacterial meningitis (figure 1, *upper-right panel,* and 1*B*).

Human PMNs activate MMP-9 via an oxidative pathway. Exposure of 2×10^6 human PMNs/mL with different concentrations (10^6–10^8 cfu/mL) of hiR6 resulted in dose-dependent release ($R^2 = 0.93$; $P < .0001$) of proMMP-9 into the supernatant, as measured by zymography (figure 2, *left panel*). Decreasing the number of PMNs resulted in a correspondingly smaller release of proMMP-9 (figure 2, *right panel*). For all subsequent experiments, we used the highest concentrations of hiR6 (10^8 cfu/mL) and PMN (2×10^6/mL) tested, levels that correspond to concentrations found in the CSF of animals that are suffering from pneumococcal meningitis. In the culture supernatants, human PMNs stimulated with hiR6 for 30 min showed a 10-fold increase in release of total MMP-9 and a 2-fold increase in release of active MMP-9, compared with unstimulated PMNs (figure 3*A*). Since ROS have been shown, in different experimental paradigms, to activate MMPs [8–10], we studied whether hiR6-triggered activation of MMP-9 is mediated by the hydrogen peroxide/myeloperoxidase system of PMNs [13]. Addition of catalase (10 μg/mL), a H_2O_2-detoxifying enzyme, significantly reduced active MMP-9 in PMN supernatants (figure 3*B*). The primary granule enzyme myeloperoxidase (MPO) transforms H_2O_2 into the more powerful oxidant hypochlorous acid (HOCl). Addition of azide (1 m*M*) inhibited activation of MMP-9, a finding that suggests that

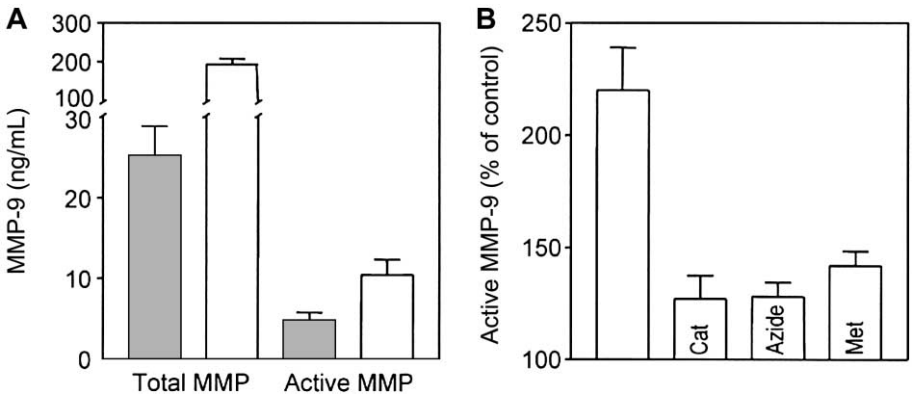

Figure 3. Oxidative activation of matrix metallo proteinases (MMPs) in vitro. *A,* Supernatants of human polymorphonuclear cells (PMNs). In supernatants of PMNs stimulated with heat-inactivated *Streptococcus pneumoniae* R6 (hiR6; *white*) for 30 min, a 10-fold increase in release of total MMP-9 ($P <$ .0001) and a 2-fold increase in active MMP-9 ($P <$ .001) was documented, compared with unstimulated control cultures *(gray). B,* Supernatants of hiR6-stimulated PMNs. In the supernatants, active MMP-9 was significantly reduced by addition of 10 μg catalase (Cat; $P <$ .001)/mL, by addition of 1 m*M* azide (Azide; $P <$ .001), an MPO inhibitor, and by addition of 1m*M* methionine (Met; $P <$ .001), a scavenger of hypochlorous acid. Therefore, MMP-9 is activated via an oxidative pathway, with the involvement of hydrogen peroxide and myeloperoxidase. Data shown result from 2 typical experiments ($n = 4$).

MPO-catalyzed HOCl formation activates MMP-9 in our system (figure 3*B*). This hypothesis is further corroborated by the finding that addition of the HOCl scavenger methionine (1 m*M*) also inhibited activation of MMP-9 (figure 3*B*). Although azide and methionine partially (~30%) inhibited the release of proMMP-9—and catalase even increased release by 30%—inhibition of proMMP-9 activation by these agents was much greater and therefore cannot be explained by a limited availability of proMMP-9.

DISCUSSION

MMPs and ROS both have been shown to be involved in BBB breakdown and in brain damage in bacterial meningitis [3, 4]. The present study was undertaken to identify the origin of MMP-9 in bacterial meningitis and to answer the question of whether ROS are involved in MMP activation. We have demonstrated that brain-slice cultures stimulated with hiR6 release proMMP-9, in the absence of invading immune cells. Brain-resident macrophages, microglia, and endothelial cells have all been shown to be sources of MMP-9 [14]. However, per microgram of cell protein and unit of time, PMNs released substantially more proMMP-9 than did brain slices and they may therefore act as a major source of MMP-9 in CSF during bacterial meningitis. This conclusion is further supported by a study of patients who were suffering from a variety of neurological conditions (including bacterial meningitis), which showed that the concentration of MMP-9 in CSF correlated significantly with the CSF cell count [15].

Our in vitro studies demonstrate that hiR6-stimulated PMNs activate MMP-9 via an ROS-dependent pathway that involves hydrogen peroxide and myeloperoxidase. This finding is consistent with other in vitro experiments using purified MMPs, which showed that HOCl oxygenates the thiol residue of the cysteine switch, thereby initiating autocleavage and activation of the enzyme [8–10]. Further studies will evaluate whether the inhibition of oxidative MMP-9 activation has the potential to protect neurons from injury in bacterial meningitis.

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